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Burn Injury Initiates a Shift in Superantigen-Induced T Cell Responses and Host Survival

Yan Zang,* Sinead M. Dolan,* Niamh Ni Choileain,* Sara J. Kriynovich,* Thomas J. Murphy,* Peter Sayles,† John A. Mannick,* and James A. Lederer2*

Severe injury induces a temporal shift in immune reactivity that can cause serious complications or even death. We previously reported that mice exposed to bacterial superantigen (SAg) early after injury undergo a strong SAg response with lethal consequences. This study compares the early and late effects of burn injury on SAg reactivity in vivo to establish how injury influences adaptive immune responses. We found that mice challenged with ordinarily sublethal doses of staphylococcal enterotoxin A or staphylococcal enterotoxin B at 1 day after burn injury exhibited high mortality, whereas no mortality occurred at 7 days after injury. This shift in mortality correlated with higher Th2-type cytokines (IL-4 and IL-10) being expressed by CD4+ and CD8+ T cells from burn as opposed to sham mice at 7 days after injury. Lymph node cells from burn-injured mice also produced higher levels of Th2-type cytokines at 7 days after injury. The results of cell-mixing studies using CD4+ and CD8+ T cells mixed with APCs from sham or burn mice suggested that changes in both T cells and APCs are involved in the altered SAg response. Finally, the biological significance of altered SAg reactivity following injury was shown by demonstrating that blocking IL-10 activity in vivo caused higher SAg-induced mortality at 7 days after injury. These findings support the idea that injury promotes a Th2-type shift in adaptive immune reactivity. Although prior studies link this counterinflammatory-type response to lowered resistance to infection, the present results suggest it may sometimes benefit the injured host. The Journal of Immunology, 2004, 172: 4883-4892.

The immune system responds to injury by rapidly producing proinflammatory cytokines and other mediators of acute inflammation. After this initial inflammatory host response, it is believed that a counterinflammatory response ensues (1-3). Although this counterinflammatory response is associated with the development of injury-induced immune suppression, it is also plausible that this response evolved to protect the injured host from the harmful effects of injury-induced inflammation. The fundamental role played by IL-10, a potent counterinflammatory cytokine, in suppressing autoimmunity and host inflammatory responses to injury and sepsis is one specific example of how the immune system adapts to control potentially harmful reactivity (4-7).

We and others have reported that severe injury causes a phenotypic shift in adaptive immune responses characterized by a sequential increase in Th2 responses as judged by the increased production of the Th2-type cytokines, IL-4 and IL-10 by polyclonal stimulated T cell populations (8-10). In most instances, this increase in Th2-type responses does not occur immediately following injury, and we also have observed that the Th2-type phenotypic switch may be preceded by an augmented Th1-type response (11, 12). First, we demonstrated that treating mice at 2 h following burn injury with a low dose of a bacterial superantigen (SAg),3 a powerful oligoclonal T cell agonist, was lethal (11). In vivo and in vitro assessment of SAg-induced cytokines at 2 or 24 h after injury showed augmented IL-2, TNF-α, and IFN-γ production. We also showed that two different strains of burn-injured TCR transgenic mice had augmented in vitro Ag-specific Th1-type cytokine production as compared with sham-injured mice if they were not immunized with cognate Ags recognized by the transgenic TCRs (12). If immunized, the burn mice, but not the sham TCR transgenic mice died of a severe shock-like response. Taken together, these findings provide evidence to suggest that burn injury augments Th1- or inflammatory-type T cell responses before causing a later switch to a Th2 phenotype.

This study addresses the interrelationship between injury-induced changes in T cell responses and the outcome of SAg administration in vivo. We show that mice treated with two different staphylococcal SAg, staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB), are susceptible to SAg-induced death if given ordinarily sublethal doses of these SAg at 1 day, but not 7 days after burn injury. We also present evidence using a newly developed in vivo SAg-induced cytokine assay to demonstrate that the shift in survival correlates with an increase in the expression of the Th2-type cytokines by SAg-stimulated CD4+ and CD8 T cells. The results of ex vivo studies confirmed that injury causes a phenotypic shift toward Th2-type cytokine responses. Moreover, we demonstrate that the injury-induced change in T cell responses against SAg involves changes in both APCs and CD4+ T cells. Finally, the in vivo significance of these findings was established by showing that burn-injured mice treated with anti-IL-10 Ab at 7 days after injury became significantly more

3 Abbreviations used in this paper: SAg, superantigen; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; BFA, brefeldin A.
susceptible to SAg-induced shock. As a whole, these results support the hypothesis that severe injury induces an exaggerated early Th1-type response against bacterial SAg, which is then followed by a compensatory Th2-type response. Additionally, these observations provide evidence suggesting that a normal change toward Th2-type responses following severe injury might protect the injured host from the lethal effects of excessive T cell activation.

**Materials and Methods**

**Animals**

Five-week-old, C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Purchased mice were acclimated for at least 1 wk before use for experiments at 6–7 wk of age. All mice were maintained in our accredited virus Ab-free animal facility in accordance with the guidelines of National Institutes of Health and the Harvard Medical School Standing Committee on Animal Research.

**Reagents**

SEA and SEB were purchased from Sigma-Aldrich (St. Louis, MO) or from Toxin Technology (Sarasota, FL). Monoclonal Abs for flow cytometry were from BD Pharmingen (San Diego, CA). ELISA Abs pairs and kits were obtained from R&D Systems (SEA, TNF-α, IL-4, IL-10, IL-6, and IL-10; Minneapolis, MN) or Caltag Laboratories (IL-2; Burlingame, CA). Brefeldin A (BFA), rat IgG, and saponin were purchased from Sigma-Aldrich. Purified anti-IL-10 mAb (JES5–2A5) produced and purified at the Trudeau Institute HybriDoma Facility (Saranac Lake, NY) was used for intravivo studies. Culture medium referred to as complete-5 (C5) was prepared by supplementing RPMI 1640 with 5% heat-inactivated FCS, 1 mM glutamine, 1 mM sodium pyruvate, 100 μg/ml nonessential amino acids, 10 mM HEPES, penicillin/streptomycin/amphotericin, and 2.5 × 10⁻⁶ M 2-ME all purchased from Life Technologies (Grand Island, NY).

**Mouse thermal injury model**

Mice were subjected to a well-established thermal injury protocol that has been approved by the National Institutes of Health and the Harvard Medical School Standing Committee on Animal Research (13). Before receiving sham or thermal injury, mice were randomized and anesthetized by i.p. injection with mixture of 125 mg/kg ketamine (Bedford Laboratories, Bedford, OH) and 6 mg/kg xylazine (Phoenix Scientific, St. Joseph, MO). Once fully anesthetized, the dorsum was shaved and mice were placed in a plastic mold that exposed 25% of their total body surface area. The mice were then subjected to scald thermal injury of the exposed skin by immersion in 90°C water for 9 s. This treatment has been shown to cause full-thickness burns in mice. Both sham and burn-injured mice were resuscitated in the same fashion except that they were exposed to room temperature (24°C) water. Both sham and burn-injured mice were resuscitated with an i.p. injection of 1 ml sterile, normal saline solution.

**Intracellular cytokine detection**

At 1 or 7 days after sham or burn injury, mice were challenged with 5 μg of SEA or 100 μg of SEB. Two hours later, inguinal, axillary, and brachial lymph nodes or spleens were harvested into C5 medium containing 10% fetal bovine serum (FBS). Tissues were then minced in C5 medium containing 10 μg/ml BFA to prevent further cytokine release. Cell suspensions were prepared and 1 × 10⁶ cells were then preincubated for 10 min with Fc block reagent in PBS containing 1% BSA and 0.1% sodium azide (PBA) in separate wells of 96-well round-bottom plates (Corning Costar, Cambridge, MA). Cells were then stained for CD4 or CD8 expression using FITC-conjugated anti-CD4 Ab or Cy5-labeled anti-CD8 Ab. Cells were washed once by centrifugation in PBA and then fixed for exactly 20 min with 100 μl of 2% paraformaldehyde in PBS (pH 7.4) at 4°C. Following fixation, cells were washed once by centrifugation with PBA, then permeabilized in 100 μl of buffer containing 0.5% saponin, 1% BSA, and 0.1% sodium azide in PBS, pH 7.4 (permeabilization buffer) (14). Cytokine staining was done by first pre-treating the fixed and permeabilized cells with 25 μl of 1 μg/ml solution of normal rat IgG (Caltag Laboratories) to block nonspecific binding of the rat anti-cytokine Abs. After 30 min, PE-labeled anti-cytokine Ab specific for IL-2, IFN-γ, IL-4, or IL-10 were added (25 μl of a 1 μg/ml solution) for an additional 30 min. Cells were washed twice with permeabilization buffer by centrifugation, then resuspended in 100 μl of PBS, pH 7.4. A FACScaliber flow cytometer (BD Biosciences, San Jose, CA) was used to detect cytokine expression levels in gated CD4⁺ or CD8⁺ T cells and the accompanying CellQuest Pro computer software was used for analysis. We collected 100,000 cell events per sample. All experiments included groups of sham or burn mice that were not given SEA or SEB and PE-labeled isotype control Abs were used to judge specific vs nonspecific staining.

**CD4⁺ and CD8⁺ T cell purification and T cell-depleted spleen cell preparation**

MACS (Miltenyi Biotec, Auburn, CA) mouse pan T cell isolation kits were used to prepare highly purified T cells by negative selection from lymph node cell suspensions following the manufacturers recommended protocols (www.miltenyibiotech.com). This whole T cell preparation was then divided into CD4⁺ and CD8⁺ T cells by selective depletions using anti-CD4 or anti-CD8 Ab coupled magnetic beads (Dynabeads; Dynal Biotech, Lake Success, NY). This approach routinely yielded highly pure (>95% pure) CD4⁺ and CD8⁺ T cell populations as judged by FACS analysis (data not shown). T cell-depleted spleen cells were prepared by treating spleen cell suspensions with anti-Thy1.2 Ab-coupled magnetic bead reagent following the manufacturers recommended protocol (Dynabeads; Dynal Biotech). In brief, after 40 min incubation at 4°C in the presence of the anti-Th1.2 magnetic beads, the T cells were removed by placing the cell suspensions in a strong magnetic field for 4 min. This protocol resulted in full depletion of both CD4⁺ and CD8⁺ T cells as judged by FACS analysis.

**In vitro lymph node and spleen cell SAg stimulation and cell mixing studies**

Groups of sham- or burn-injured mice were sacrificed at 1 and 7 days and their axillary, brachial, and inguinal lymph nodes or spleens were harvested into cold C5 medium. These organs were minced using sterile fine-meshed stainless steel screens to obtain single cell suspensions. Following two washes by centrifugation at 300 × g for 10 min in C5 medium, lymph node and spleen cell suspensions were counted using a hemacytometer, diluted in C5 medium, and subsequently plated at a density of 5 × 10⁴ cells per well of a 96-well round-bottom tissue culture plate (Corning Costar). These cell preparations were cultured at 37°C in 5% CO₂ for 48 h in the absence or presence of SEA (200 ng/ml). For cell mixing studies, purified CD4⁺ or CD8⁺ T cells (1 × 10⁵ cells) were cultured with T cell-depleted spleen cells (4 × 10⁵ cells) in wells of a 96-well round-bottom plate in the absence or presence of SEA (200 ng/ml). Cell mixtures were prepared to compare injury-matched with injury-mismatched spleen cell and T cell combinations. After 48 h incubation at 37°C, 5% CO₂, supernatants were harvested and tested for IL-2, IFN-γ, TNF-α, IL-4, IL-6, or IL-10 levels by ELISA.

**Cytokine ELISA**

ELISA using Ab pairs specific for IL-2, IFN-γ, TNF-α, IL-4, IL-6, and IL-10 was used to assess relative SAg-stimulated cytokine levels in 48-h culture supernatants. They were performed using a conventional sandwich technique following the manufacturers protocols (R&D Systems; www. endogen.com). Serial dilutions of cytokine standards and the unknown samples were added to individual wells in triplicate and upon completion of the ELISA protocol, an ELISA plate reader (Molecular Devices, Sunnyvale, CA) and its accompanying computer software program SOFTmax PRO version 1.1 were used to analyze the results.

**Statistical analysis**

The Prism 3.0 statistical software program (GraphPad, San Diego, CA) was used for statistical analysis of data. The statistical tests used included two-tailed paired t test and the log rank or Fisher’s exact test for survival studies. Values of p < 0.05 were considered statistically significant.

**Results**

**Injury elicits a significant increase in susceptibility to SAg-induced lethality at 1 day, but not 7 days after injury**

Groups of sham- or burn-injured mice were challenged by i.p. injection with SEA or SEB at either 1 or 7 days after injury with doses of these SAgs predetermined to be sublethal in normal, unmanipulated mice. Survival was then monitored daily for 7 days after SAg challenged. As shown in Fig. 1A, a significant fraction of burn-injured mice succumbed to the lethal effects of either SEA or SEB treatment, whereas sham mice showed no mortality. Moreover, all burn-injured mice that were challenged with these SAgs at this time point displayed visible signs of distress by 1 day after SAg challenge, whereas sham-injured mice appeared unaffected.

...
(our unpublished observations). The SEA-induced mortality was significant by both the log rank and Fisher’s exact statistical tests, whereas SEB-induced mortality was significant only by the Fisher’s exact test. In marked contrast, burn-injured mice given these same doses of SEA or SEB at 7 days after injury showed no significant increase in mortality and no visible evidence of SAg-induced distress (Fig. 1B). These findings clearly demonstrate that burn-injured mice underwent a phenotypic change in their reactivity to SAg challenge from a lethal early response to a nonlethal response at 7 days after injury.

Injury causes a phenotypic change in CD4+ and CD8+ T cell cytokine responses

To address whether survival was associated with specific changes in T cell SAg reactivity in vivo, we developed an approach that allowed us to directly compare cytokine expression in CD4+ and CD8+ T cells at 1 vs 7 days after SAg challenge. Specifically, we used intracytoplasmic cytokine detection by flow cytometry to visualize cytokine expression profiles within CD4 and CD8 T cells harvested directly after challenging mice with SEA or SEB. Preliminary studies indicated that the success of this approach depended on the inclusion of BFA, a protein transport inhibitor, into the tissue and cell harvest medium to halt cytokine secretion by T cells activated in vivo by SAg. Moreover, time course studies designed to optimize this experimental approach showed that harvesting lymph nodes or spleens at 2 h following SAg challenge provided peak cytokine detection in CD4+ and CD8+ T cells. Using these optimal experimental conditions, sham- or burn-injured mice were injected with SAg (SEA or SEB) and 2 h later their lymph node and spleen CD4 and CD8 T cells were stained for IL-2, IFN-γ, TNF-α, IL-4, and IL-10 expression. The representative intracellular FACS plots in Fig. 2 are included in this report to illustrate that this technique allowed us to visualize SAg-induced cytokine expression in both CD4 and CD8 T cells. Mice that were not injected with SAg served as controls for background cytokine staining. An important outcome of these cytokine-staining experiments is the demonstration that SAg has the capacity to induce cytokine responses in both CD4+ and CD8+ T cells. Furthermore, we discovered an interesting dichotomy in the class of cytokines expressed by CD4+ and CD8+ T cells following SAg challenge. CD4 T cells expressed predominantly IL-2 and TNF-α, whereas CD8 T cell preferentially expressed IFN-γ following SEA or SEB treatment. This was true for cells prepared from either the lymph nodes or spleens.

The results comparing SEA- or SEB-induced cytokine responses in the lymph nodes and spleens of sham or burn mice at 1 vs 7 days after injury are shown in Figs. 3 or 4, respectively. Comparing the cytokine staining patterns for CD4+ T cells at 1 day vs 7 days after injury revealed a shift toward increased Th2-type (IL-4 and IL-10) cytokine expression following SEA or SEB treatment. This phenotypic shift toward higher SAg-stimulated Th2-type cytokine expression was most apparent in splenic CD4+ T cells. The increased Th2-type reactivity observed in splenic T cells from SAg-challenged mice was in contrast to the lower IL-4 and IL-10 expression by SAg-activated CD4+ T cells observed at 1 day after injury. The cytokine staining profiles of CD8+ T cells at 1 vs 7 days after injury were also different. At 7 days after injury, CD8+ T cells expressed significantly higher levels of IL-4 and IL-10 following SEB challenge as compared with 1 day after injury. A similar trend was observed in experiments using SEA challenge, but the shift was not as apparent. To our surprise, we did not observe significant increases in Th1-type cytokine expression by CD4+ or CD8+ T cells at 1 day after injury. Taken together, these results demonstrate that burn injury promotes a late increase in Th2-type cytokine expression by CD4 and CD8 T cells.

Burn injury induces compartmentalized changes in lymph node and spleen cytokine production profiles

Although intracytoplasmic staining is an efficient method for identifying which cells express a particular cytokine, it provides no measurement of cytokine release. To obtain this information, we prepared lymph node or spleen cell suspensions from sham- or burn-injured mice and stimulated them ex vivo with SEA or SEB after which culture supernatants were tested for cytokine levels by ELISA. The results presented in Fig. 5, support our in vivo findings with respect to the skewing toward increased Th2-type cytokine production by SAg-stimulated T cells a 7 days after injury. At 7 days after burn injury, we observed a significant injury-induced increase in IL-4, IL-6, and IL-10 production by SEB-stimulated lymph node cells, whereas at 1 day after burn injury lymph node cells produced significantly higher levels of IL-2 and IFN-γ than cells from sham mice. By 7 days after injury, this increase in SAg-induced IL-2 and IFN-γ disappeared. Collectively, these results suggest that injury caused a heightened Th1-type response at 1 day and an increased Th2-type response at 7 days after injury. However, SAg-stimulated spleen cells did not as clearly follow this scenario. Rather than increased IL-4 and IL-10 production at day 7 after burn injury, SAg-stimulated spleen cells demonstrated a significant decrease. However, we did observe a significant burn-induced increase in IL-6 production at both 1 and 7 days after injury. In contrast to our lymph node results, IFN-γ was the only Th1-type cytokine whose production was significantly increased at 1 day after burn injury. Not surprisingly, these data suggest that
burn injury differentially influences adaptive immune reactivity in these major secondary lymphoid compartments.

The injury-induced change in SAg responses involves phenotypic changes in both APCs and T cells

Next, we were interested in determining whether these described injury-induced changes in SAg-stimulated T cell responses were dependent upon APCs or T cells. To accomplish this, we performed a series of cell mixing studies. First, we mixed burn or sham APC populations with injury-matched highly purified CD4+ or CD8+ T cells to determine whether CD4+ or CD8+ T cells were the major source of Th2-type cytokines. As shown in Fig. 6, CD4+ T cells stimulated with SEA by their injury-matched APC populations produced IL-2, IL-4, IL-10, and low levels of IFN-γ, whereas CD8+ T cells produced mostly IFN-γ when stimulated by their injury-matched APCs. This finding correlates with the SAg-stimulated intracytoplasmic cytokine staining profiles. Similar results were obtained using SEB (data not shown). Direct comparison between 1- and 7-day results revealed that CD4+ T cells from burn mice cultured with APCs from burn mice produced higher levels of IL-4 and IL-10 at 7 days after injury than CD4+ T cells from sham mice. In contrast, CD8+ T cells from burn mice did not display any significant changes in SAg-induced cytokine production when cultured with burn APCs at 1 or 7 days after injury.

Studies were then performed using APC populations prepared from sham- or burn-injured mice mixed with injury-mismatched CD4+ or CD8+ T cells. The results of these experiments revealed that APCs prepared from sham mice did not mediate increased Th2-type cytokine production by SAg-stimulated CD4+ or CD8+ T cells from burn-injured mice (Fig. 7). Conversely, APCs prepared from burn-injured mice mediated a significant increase in IL-10 production by burn CD4+ T cells as compared with sham CD4+ T cells. In addition, CD4+ T cells from sham-injured mice did not demonstrate increased SAg-stimulated IL-10 production when activated with burn APCs. Surprisingly, we failed to detect any significant injury-related changes in cytokine production profiles by CD8+ T cells stimulated under these same experimental conditions. All in all, the results of these cell mixing experiments suggest that combined injury-induced changes in APCs and CD4+ T cells mediate the phenotypic shift toward increased Th2-type responses at 7 days after injury.

**IL-10 mediates protection from SAg-induced shock late after injury**

To determine the relationship between the phenotypic shift in SAg responses and host survival, we performed another set of SAg-induced mortality studies testing the effects of blocking IL-10 on host survival. As shown in Fig. 8, burn-injured mice treated with 0.25 mg of anti-IL-10 Ab 1 h before SEA challenge exhibited a significant increase in mortality. This suggests that anti-IL-10 treatment restored an injury-related lethal SAg-response cascade. Importantly, we did not observe any SEA-mediated mortality in the sham-injured mouse group that was treated with anti-IL-10 Ab. In total, these observations support the idea that IL-10 protects mice from SAg-induced death at 7 days after injury.

**Discussion**

These experiments were initiated to test the hypothesis that a phenotypic shift toward increased Th2-type immune reactivity following severe injury might serve to protect the host from undergoing excessive and potentially harmful T cell-mediated immune responses against microbial or self-Ags. Prior observations showing that burn-injured mice undergo a lethal shock-like response when challenged with SAg at 2 h after injury prompted this line of investigation (11, 14). The results of a separate study showed that immunizing two different TCR transgenic mouse strains at the time of injury with peptides specific for the transgenic TCRs resulted in 100% mortality, whereas none of the sham-injured mice died (12).
Together, these findings suggested to us that burn injury provokes an early strong and vigorous T cell-mediated inflammatory response. Rather than pursuing the transgenic TCR experimental system for this work, we elected to perform studies using bacterial SAgs to confirm and elucidate this phenomenon. Although our intent in this study was to use SAgs as experimental tools, there is some limited evidence to suggest that they may contribute to complications in critically injured patients. In fact, a recent report demonstrated a genetic link between the strength of streptococcal SAg reactivity and the severity of the systemic inflammatory response occurring as a result of invasive streptococcal infections (15). Also, many pathogens associated with opportunistic infections that occur in critically injured patients do have the capacity to produce SAgs (16–18). Unfortunately, the relative incidence of SAg-related mortality in critically injured patients is not known (19).

As their name implies, SAgs are potent oligoclonal T cell stimulants that activate CD4 and CD8 T cells in an MHC class II-restricted fashion via specific interaction with Vβ chains of T cell receptors (20). They cause a potent and synchronous activation of T cells bearing the appropriate TCR Vβ chain and if the response is excessive, a lethal shock-like response occurs that is mediated in part by cytokines (21, 22). We initially tested the effect of burn injury on SAg-induced responses in the FVB mouse strain (11). One objective of this present study was to confirm that we could reproduce our original findings in C57BL/6J mice. One advantage of using C57BL/6J mice for SAg-based experiments is that they display high reactivity to two common staphylococcal SAgs, SEA and SEB, whereas FVB mice appeared to respond to only SEA (our unpublished observations). An additional advantage of adapting this experimental model to the C57BL/6J mouse is that a wide variety of genetically modified mice are readily available in this background strain. Our first study tested whether C57BL/6J mice would succumb to SAg challenge in an injury-dependent fashion as was reported previously (11). We were encouraged to find that C57BL/6J mice did indeed display a significant increase in mortality to SEA or SEB challenge at 1 day after burn injury. It was
also apparent that SEA and SEB had differing shock-inducing potency in burn-injured mice. SEA had to be given at a higher dose than SEB to induce mortality and we found that SEA was at least 5-fold more potent in C57BL/6J mice than SEB in our ex vivo studies. Overall, we were pleased to find that the injury-induced susceptibility to SAg responses was not restricted to one mouse strain or a particular SAg. This supports the idea that a variety of endogenous or exogenous molecules with SAg or SAg-like activity could provoke a similar reactivity following injury (23, 24).

Next, we wanted to determine whether burn-injured mice exhibit a similar difference in mortality if challenged with the same doses of these SAg at 7 days after injury, a time point when injured mice are known to express a Th2 phenotype (8, 10, 25). We found that burn-injured mice did not die when given either SEA or SEB at 7 days after burn injury at doses that were fatal at 1 day after injury. This observation indicates that these mice underwent a phenotypic change in SAg reactivity and that this change provided protection from the lethal consequences of SAg challenge. Because the initiation of SAg responses requires direct interaction between MHC class II-bearing APCs and the appropriate TCR Vβ chain expressed on T cells, we conclude from the results of our mortality studies that injury might cause an early enhanced, inflammatory and a later counterinflammatory T cell-mediated immune response, whereby the counterinflammatory response is protective (26, 27).

Because we and other groups have described that injury causes a delayed increase in Th2-type immune responses, we focused on determining whether the change in SAg-mediated lethality was associated with an increase in Th2-type responses (8, 10, 28). To test this possibility, we developed an experimental approach that allowed us to directly assess how injury alters SAg-induced T cell cytokine expression immediately after SAg challenge. Our first attempts to detect cytokine expression profiles in CD4+ and CD8+ T cells following in vivo SAg treatment were based upon a single report showing that this was indeed possible (29). In that report, it was shown that lymph node or spleen cells could be harvested from mice after SAg challenge and cytokines could be visualized by intracytoplasmic staining with cytokine-specific Abs. However, this initially proved difficult due to the low level of cytokine detection and high variability among SAg-treated mice. We reasoned
that if we could halt the release of cytokines from activated T cells, we would be able to enhance their intracellular detection and decrease variability due to differences in processing time between samples. Thus, we assessed whether inclusion of BFA, a drug that blocks cytokine release, into the tissue and cell harvest medium would help increase detection levels and reduce variability. We found that this approach yielded high-quality staining with increased cytokine staining levels and low mouse-to-mouse variability. In addition, we were able to detect intracellular IL-4 and IL-10, which tend to be expressed at comparatively lower levels than Th1 cytokines following SAg stimulation (26).

Using this technique, we made several significant observations. First, we showed that both CD4+ and CD8+ T cells rapidly respond to SAg stimulation with distinct cytokine expression patterns. Initially, we found this result surprising because it is known that SAgs initiate T cell responses by binding to MHC class II molecules, and CD4+ T cells are MHC class II restricted, whereas CD8+ T cells respond in an MHC class I-restricted fashion (30). However, we clearly demonstrate by intracellular staining and by studies using purified CD8+ T cells that these cells respond efficiently to SAg in vivo and in vitro. Perhaps, the Vβ TCR chains present on CD8+ T cells are responding directly to MHC class II-SAg complexes in an unconventional MHC class I-independent fashion as has been suggested by other investigators (31, 32). We acknowledge that this is only one potential explanation for our findings and that the activation of CD8+ T cells by SAg is a not a well-described or understood phenomenon.

Comparing cytokine expression profiles in CD4+ and CD8+ T cells at 1 vs 7 days after injury in SAg-treated mice showed that injury caused a significant qualitative change in the types of cytokines expressed by CD4+ and CD8+ T cells. Overall, we found an increase in expression of the Th2-type cytokines by CD4+ and CD8+ T cells at 7 days after injury. This supports the hypothesis that injury causes a natural phenotypic switch in the immune system toward increased Th2-type reactivity. This type of shift in immune reactivity against bacterial SAgs has been described in a number of studies in which mice were prechallenged or chronically challenged with high doses of SAg (33, 34). These investigators observed a similar increase in Th2-type cytokine production and this increase correlated with reduced SAg-induced proliferation and, in some instances, suppressed IL-2 and IFN-γ production. Those studies were interpreted as suggesting that SAg exposure could induce a form of tolerance to rechallenge with SAg. Considering our observations together with those documenting SAg-induced tolerance, one could reason that injury might induce changes in the host immune response mimicking SAg reactivity. This would mean that injury might share some of the properties of strong SAg-induced responses. Although speculative, stress, increased bacterial translocation from the gastrointestinal tract, or the tissue necrosis associated with burn injury are potential sources of injury related SAg-like activity.

The cell mixing studies were designed to test if the shift toward increased Th2 cytokine production was due primarily to changes in APCs or T cells. The APCs were prepared from the spleens of sham- or burn-injured mice by depleting T cell from spleen cell suspensions using anti-Th1.2 Ab-coupled magnetic beads. This approach effectively eliminated both CD4+ and CD8+ T cells and thus made it possible to examine APC-dependent changes in SAg-induced cytokine production by purified CD4+ or CD8+ T cell populations from sham- or burn-injured mice. Our findings support several important conclusions. First, we found that CD4+ T cells produced higher levels of IL-2 and TNF-α than CD8+ T cells and CD8+ T cells produced higher levels of IFN-γ than CD4+ T cells. This observation fully supports the intracytoplasmic cytokine staining results showing differential expression of the SAg-induced cytokines in CD4+ and CD8+ T cells. Next, we found that CD4+
T cells were the primary source of increased SAg-induced IL-4 and IL-10 production at 7 days after burn injury and that the combination of burn APCs and burn CD4^+ T cells was required for the full expression of this enhanced Th2-type phenotypic shift. Although this finding adds complexity to our understanding of how injury modulates host immunity, it suggests that injury promotes phenotypic changes in cells of the innate and adaptive immune systems. Moreover, the discovery that CD4^+ T cells are the primary cell type expressing the Th2-type response suggests that the initiation of the phenotypic switch in SAg-stimulated T cell reactivity may involve an injury-specific MHC class II-restricted CD4^+ T cell response. This idea is supported by the findings of a recent report that uses a TCR transgenic mouse adoptive transfer model to demonstrate that CD4^+ T cells do not acquire a Th2-type phenotype following burn injury unless they are exposed to their cognate Ag (35).

We have shown that mice exhibit a time-dependent shift toward increased Th2-type SAg reactivity and that this correlates with increased survival to doses of SEA or SEB that are otherwise lethal when given at 1 day after injury. This dichotomy in SAg lethality between 1 and 7 days after injury inspired us to attempt to reverse the survival phenotype displayed by mice at 7 days after burn injury. Our available data on cytokine production suggested to us that IL-10 might be involved in protecting mice from SAg-induced lethality at 7 days post-injury. Thus, we chose to block the potential protective effects of IL-10 by treating 7-day burn mice with anti-IL-10 Ab just before SEA challenge. As shown, we observed a significant increase in SAg-induced death when burn-injured mice were treated with anti-IL-10 Ab. The mortality in the anti-IL-10 Ab-treated burn mice was not nearly as high as what was observed at 1 day after injury, but nevertheless suggests that IL-10 contributes to the protection from the lethal effects of SEA challenge. A role for IL-10 in protecting mice from SAg-induced shock is not unprecedented because it was demonstrated that treating mice with IL-10 Ab just before or at the time of SEB challenge significantly protected them from SEB-induced lethal shock (36). However, the present study provides the first evidence to suggest that IL-10 may actively control the level of T cell reactivity following injury and as such provides a link between the development of a Th2-type phenotype following injury and protecting the injured host from potentially excessive T cell reactivity.
for IL-2, IFN-γ, IL-4, IL-10 levels by ELISA. The results are plotted as the mean ± SEM of three to four independent experiments using three mice per group. *, p < 0.05 sham vs burn by a two-tailed paired t test.

FIGURE 7. Mismatched APCs studies using CD4+ and CD8+ T cells from mice at 7 days after sham or burn injury. Lymph node CD4+ and CD8+ T cells were purified from sham- or burn-injured mice at 7 days after injury, then mixed with T cell-depleted spleen cells (APCs) from 7-day sham or burn mice to obtain injury mismatched cell cultures. After 48 h stimulation with 200 ng/ml SEA, supernatants were harvested and tested for IL-2, IFN-γ, IL-4, or IL-10 levels by ELISA. The results are plotted as the mean ± SEM of three to four independent experiments using three mice per group. *, p < 0.05 sham vs burn by a two-tailed paired t test.

In contrast, experiments performed by us and other groups have shown that preventing the development of a post-injury Th2-type response by early treatment with Th1-promoting factors like IL-12, IL-18, or anti-IL-10 mAb protects mice from dying in response to later cecal ligation and puncture-induced polymicrobial sepsis, Escherichia coli-induced sepsis, or viral infection (37–41). The collective outcomes from these studies suggest that promoting a Th1-type response protects mice from developing post-injury immune suppression as judged by increased resistance to infectious challenge. However, in this study, we addressed the effects of injury on host immunity from a different perspective investigating an alternative reason for a shift in adaptive immune responses toward a counterinflammatory Th2-type phenotype. Although we show that an enhanced Th2-type response correlates with improved survival following SAg challenge, we do not suggest that injury-induced changes in T cell-mediated immune function are necessarily protective. Instead, these results suggest that the counterinflammatory nature of the T cell response can potentially protect the host from excessive T cell reactivity if infectious complications do occur. In a sense, the observation that treating burn-injured mice at 7 days after injury with anti-IL-10 Ab can be detrimental, whereas anti-IL-10 Ab treatment during the early time period after injury protects mice from cecal ligation and puncture-induced mortality later after injury, highlights this idea. Blocking early IL-10 activity after injury prevents the development of immune dysfunction and helps the injured host resolve infections and prevents sepsis, whereas later IL-10 inhibition boosts this T cell-mediated inflammatory response to a dangerous and lethal level. In closing, we have shown that burn injury promotes a natural shift in the adaptive immune response toward a Th2 phenotype. This Th2 shift protected mice from SAg-induced lethality suggesting that severe injury initiates an immune response that ultimately serves to protect the host from excessive T cell activation. We provide evidence that the counterinflammatory cytokine, IL-10, plays a role in this protective host response. Thus, we speculate that this acquired injury-induced change in T cell-mediated reactivity may have evolved as a mechanism to help counteract the harmful inflammatory responses initiated by injury or infection. This host response scenario has clinical implications because an unfortunate consequence of a strong Th2-type immune shift following injury is the development of a transient state of immune suppression putting the injured host at higher risk of developing opportunistic infections (8, 41, 42). This “double-edged sword” nature of the biased Th2-type immune response following injury is one example of the complexity involved in the attempt by the mammalian immune system to maintain immune homeostasis after severe injury. Future research efforts will be aimed at improving our understanding of how injury promotes this phenotypic change.

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References

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